AWARD NUMBER: W81XWH-16-1-0134

TITLE: Systemic Gene Therapy for Tuberous Sclerosis

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REPORT DATE: July 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2017	Annual	1JUL2016 - 30JUN2017

4. TITLE AND SUBTITLE

Systemic Gene Therapy for Tuberous Sclerosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0134

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Boston, MA 02114-2554

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Tuberous sclerosis complex (TSC) patients suffer from hamartomas in various organs, including subependymal nodules in the brain, angiomyolipomas in the kidney and liver, and lymphaganioleiomyomatosis (LAM) of the lungs leading to a range of symptoms, some of which are life-threatening. Current drug treatment has some limitations and there is a pressing need to develop new therapies, especially for children and LAM patients. Our group is focused on developing gene therapy for TSC which has the potential for single application and low-to-no toxicity with genetic replacement of the deficient protein in multiple tissues. We have generated mouse models of TSC1 (hamartin deficiency) and TSC2 (tuberin deficiency) by adeno-associated virus (AAV) delivery of Cre recombinase into Tsc1 floxed mice into the brain and kidney, and by xenograft transplantation of human TSC2 LAM cells into immune compromised mice, respectively. In the Tsc1 model we have shown that intravascular (IV) delivery of an AAV vector encoding hamartin can lead to prolonged survival and normalization of neuronal cell size in the brain. We have also created a condensed version of tuberin, such that the cDNA encoding it fits into an AAV vector. Initial experiments show that injection of this AAV-cTuberin vector into subcutaneous LAM tumors in immune compromised mice curtails tumor growth. AAV has proven to be safe and beneficial in Phase 1 clinical trials for neurologic diseases in adults and children, and AAV9 can deliver genes not only to peripheral tissues, but also to the brain in mice and non-human primates following IV delivery, with transgene expression extending for years.

15. SUBJECT TERMS gene therapy, tuberous sclerosis, mouse model, hamartin, tuberin, brain ventricles, AAV vectors, LAM tumors

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	UU	11	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Tuberous sclerosis complex (TSC) patients suffer from hamartomas in various organs, including subependymal nodules in the brain, angiomyolipomas in the kidney and liver, and lymphangioleiomyomatosis (LAM) of the lungs leading to a range of symptoms, some of which are life-threatening. Current drug treatment has some limitations and there is a pressing need to develop new therapies, especially for children and LAM patients. Our group is focused on developing gene therapy for TSC which has the potential for single application and low-to-no toxicity with genetic replacement of the deficient protein in multiple tissues. We have generated mouse models of TSC1 (hamartin deficiency) and TSC2 (tuberin deficiency) by adeno-associated virus (AAV) delivery of Cre recombinase into *Tsc1* floxed mice into the brain and kidney, and by xenograft transplantation of human TSC2 LAM cells into immune compromised mice, respectively. In the *Tsc1* model we have shown that intravascular (IV) delivery of an AAV vector encoding hamartin can lead to prolonged survival and reduction of neuronal cell size in the brain. AAV has proven to be safe and beneficial in Phase 1 clinical trials for neurologic diseases in adults and children, and AAV9 can deliver genes not only to peripheral tissues, but also to the brain in mice and non-human primates following IV delivery, with transgene expression extending for years.

2. KEYWORDS

gene therapy, tuberous sclerosis, mouse model, hamartin, condensed tuberin, brain ventricles, AAV vectors, LAM tumors

3. ACCOMPLISHMENTS

What are the major goals of the project? Our studies are designed to evaluate whether gene replacement therapy for hamartin and tuberin deficiency can provide therapeutic benefit by extending lifespan and reducing lesion size in brain, kidney and lungs in mouse models of TSC1 and TSC2.

Specific Aim 1: Gene replacement therapy in systemic, stochastic mouse model of TSC1.

Major Task 1: Evaluate therapeutic effects of AAV-hamartin injections in stochastic *Tsc1* mouse model. 50% complete

Major Task 2: Carry out pathological analyses on tissues from therapeutic trial in stochastic *Tsc1* model. 50% complete

Specific Aim 2: Gene replacement therapy in a LAM model of TSC2.

Major Task 3: Generate and test condensed version of tuberin construct. 90% complete

Major Task 4: Evaluate therapeutic effects of AAV-cTuberin injections in Tsc2 LAM mouse model. 30% complete

What was accomplished under these goals?

Aim 1 -

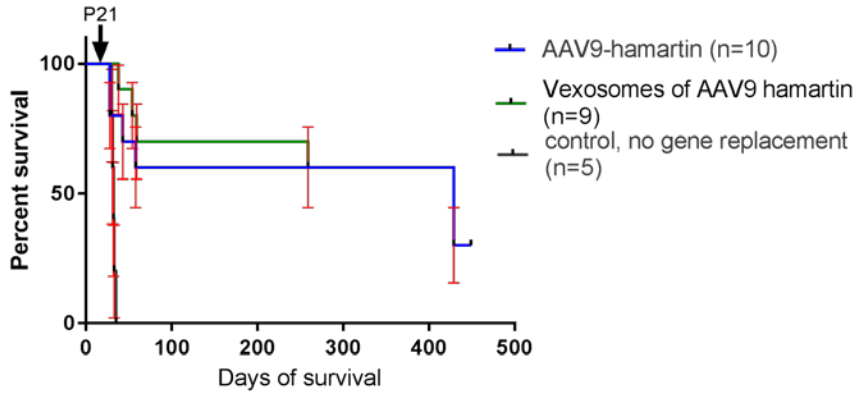
Major activities: Tuberous sclerosis complex (TSC) is an autosomal-dominant disorder caused by second hit somatic mutations in tumor suppressor genes, *TSC1* or *TSC2*, encoding hamartin or tuberin, respectively. These proteins act as a complex which inhibits mTOR-mediated cell growth and proliferation. Loss of either protein leads to overgrowth of cells in many organs, most commonly affecting the brain, kidneys, skin, heart and lung

For our Tsc1 mouse model we used mice homozygous for floxed *Tsc1* alleles such that expression of Cre recombinase delivered by an AAV vector results in loss of hamartin in transduced cells (Prabhakar et al., 2013). In this stochastic mouse model of TSC brain lesions, complete loss of hamartin is achieved in multiple cell types in the brain by injection of an AAV vector encoding Cre recombinase into the cerebral ventricles of mice homozygous for *Tsc1*-floxed alleles on the day of birth (P0), leading to reduced survival (mean 32 days), and brain pathologic findings consistent with TSC (*ibid).* These Cre-injected mice were then injected with an AAV-hamartin vector or control vector intravenously on day 21 (P21) and the behavior and lifespan of these mice was followed. Mice were also sacrificed for immunocytochemistry and histopathological analysis.

Specific objectives: To see if AAV-mediated gene replacement therapy of hamartin could increase the health and survival time in this Tsc1 mouse model.

Key outcomes: For vector injections, on the day of birth (P0), $Tsc1^{c/c}$ROSA (Prabhakar et al., 2013) neonates were cryo-anesthetized and injected with 2 µl of viral vector AAV1-CBA-Cre of titer 6.97 x 10^{12} genome copy (g.c.) per 1 µl into each cerebral lateral ventricle (ICV) with a glass micropipette (70–100 mm diameter at the tip) using a Narishige IM300 microinjector at a rate of 2.4 psi/sec (Narshige International, East Meadow, NY). Mice were then placed on a warming pad and returned to their mothers after regaining normal color and full activity typical of newborn mice. *Note.* The CBA promoter is a strong promoter active in most cell types.

At 3 weeks of age (P21), the same mice were anaesthetized with isoflurane inhalation (3.5% isoflurane in an induction chamber and then maintained anesthetized with 2-3% isoflurane and 1-2 liter/min $O_2$for the duration of the experiment), AAV vectors were injected retro-orbitally into the vasculature right behind one of the eyeballs in a volume of 70 µl (10 µl of AAV9 + 60 µl 0.9% saline) using AAV9-hamartin-cmyc (titer 2.4E13 g.c./ml or the same vector packaged in extracellular vesicles (evAAV, titer 1.5E13 g.c./ml) or non-injected (as controls) using 0.3 ml insulin syringe over less than 2 min (Yardeni et al., 2011). *Note*. C-myc is an antigenic tag to distinguish hamartin encoded in AAV from endogenous hamartin in the mice.



Median survival :
TSC1 ROSA w/ AAV1-Cre @ P0,2ul; RO w/ AAV9-hamartin @ P21(n=10) : 429 days
TSC1 ROSA w/ AAV1-Cre @ P0,2ul; RO w/ vexosomes of AAV9-hamartin @ P21(n=9) : 429 days
TSC1 ROSA w/ AAV1-Cre @ P0,2ul; no gene replacement (n=5) : 32 days
Log-rank (Mantel-Cox) test: P=0.0002, significant
Gehan-Breslow-Wilcoxon test: P=0.0007, significant

Fig. 1. Gene replacement is able to increase survival of TSC1c/c mice injected with AAV-CBA-Cre vectors. TSC1c/c pups were injected into both cerebral ventricles with the AAV1-CBA-Cre vector at P0 (day of birth). This procedure has previously been shown to produce subependymal nodules and death by hydrocephalus by around P60 (Prabhakar et al., 2013). At P21 days, 10 mice were injected with AAV9-hamartin, 13 mice with vexosomes of AAV9-hamartin into the retro-orbital vein in the eye (left eye), and 13 mice were only injected at P0 with the AAV1-CBA-Cre vector and no retro orbital injections at P21. Survival curve based on the Log-rank (Mantel-Cox) Test with a statistical significance between groups.

ICV injections were carried out in several litters of $Tsc1^{c/c}$ROSA mice, followed by the retro-orbital injections (as above) to monitor survival and a few mice were sacrificed at certain intervals, sent to the Rodent

Histopathology laboratory at the Harvard Medical School for the histopathologic analysis. Brain tissues have also been prepared for immunocytochemistry for c-myc, pS6, NeuN and GFAP (ongoing).

To test if overexpression of hamartin is toxic, we are doing toxicity studies by injecting AAV9-hamartin in two dosages (10 µl and 20 µl to a group of 3 mice each) at P21 via retro-orbital vasculature, waiting for 3 weeks and sacrificing the mice for histopathology (ongoing).

Remarkably when these mice were injected intravenously on day 21 (P21) with an AAV vector encoding hamartin, they survived over (429) days in an apparently healthy condition (**Fig. 1**) with marked reductions in brain pathology (ongoing). *Note.* Normal laboratory mouse lifespan is about 600 days. As **major findings** we show that systemic gene replacement through the vasculature at an early age replaces enough of the missing hamartin so as to overcome the detrimental effects of loss of hamartin in brain cells, and that overexpression in cells that had not lost hamartin, had no overt toxicity. This gene therapy approach has the advantages that therapy can be achieved from a single application, as compared to repeated treatment with drugs, and that AAV vectors have been found to have minimal to no toxicity in clinical trials for other neurologic conditions.

Other achievements: Nothing to report

Aim 2 –

Major activities: In Aim 2 we explored use of an AAV vector encoding tuberin to reduce lesions in a mouse model of TSC2. Unfortunately, the size of tuberin cDNA exceeds the packaging capacity of AAV. Therefore, we engineered a condensed form of the tuberin cDNA (cTuberin) encoding discreet functional domains of the parent protein, which fits into an AAV vector, and injected it into subcutaneous tumors formed in nude mice following injection of immortalized LAM cells from a TSC2 patient (Yu et al., 2003; Hong et al., 2008), which we obtained from Dr. Elizabeth Henske at Brigham and Women's Hospital (BWH). We stably transduced these cells with an expression cassette for firefly luciferase so that we could monitor growth of the tumors by *in vivo* bioluminescence imaging.

Specific objectives: To see is a condensed form of tuberin encoded in an AAV vector and injected into LAM tumors in mice would curtail tumor growth.

Key outcomes: With insert capacity of AAV vectors of about 4.7 kb (including promoter, transgene, polyA sequence and other regulatory elements), the cDNA for hamartin (1.5 kb) can easily be accommodated, while that for tuberin (5.4 kb) cannot. In order to generate a potentially functional form of tuberin encoded in a shorter cDNA (2.3 kb), we generated a condensed version (cTuberin) containing the N-terminal domain which binds to hamartin and the C-terminal domain that has GAP (GTPase-activating protein) activity (**Fig. 2**) and inserted this into an AAV construct under a CBA promoter (**Fig. 3, Fig. 4**).

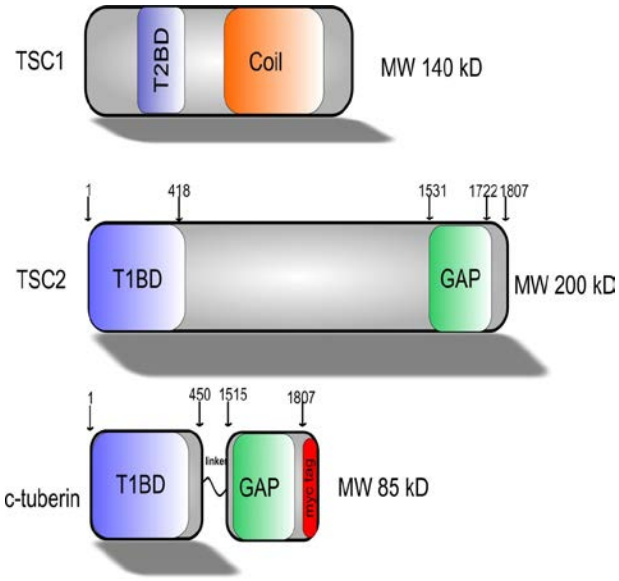


Fig. 2. Schematic of the TSC1, TSC2 and cTuberin proteins. The functional domains on TSC1 and TSC2 are depicted schematically with numbers representing amino acid residues on the full-length human proteins. Abbreviations: T2BD, TSC2-binding domain; T1BD, TSC1-binding domain; Coil, predicted coiled-coil domain; GAP, GAP domain homologous with that in Rap1GAP.

In an initial experiment we also found that injection of the AAV9-cTuberin vector (titer 3 x 10^{12} g.c./ml) into subcutaneous LAM tumors curtailed their growth (**Fig. 5**).

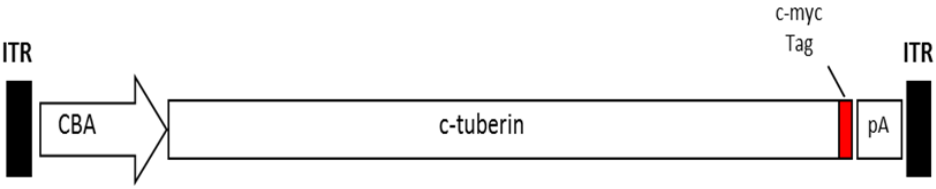


Fig. 3. AAV-CBA-cTuberin-cmyc construct. AAV cassette in AAV2-LTR backbone in which a condensed version (cTuberin) containing the N-terminal domain which binds to hamartin and the C-terminal domain with GAP (GTPase-activating protein) activity encoded in a cDNA of 2.3 kb which fits into an AAV vector under CBA promoter and tagged with a c-Myc peptide at the C-terminal. pA refers to polyadenylation signal.

Major findings to date are that we are able to express the cTuberin protein within the context of the AAV vector construct and that injection of this vector into subcutaneous TSC2 LAM tumors appears to arrest their growth (single experiment to date; ongoing).

Other achievements: Nothing to report.

What opportunities for training and professional development has the project provided?

We have met with clinicians (Drs. Elizabeth Thiele, Florian Eichler and Scott Plotkin) and their staff at Massachusetts General Hospital (MGH) on several occasions to go over our current data and to discuss how to proceed with our preclinical studies in a way that would be most useful in evaluating the potential of this therapeutic strategy in TSC patients.

Dr. Breakefield also gave a lecture on "Gene therapy for neurologic diseases" including preclinical gene therapy for TSC to students in the MGH Summer Scholar program.

How were the results disseminated to communities of interest?

Oral presentations with power point images have been given to our MGH Gene Therapy Working Group and the Harvard Medical School TSC Symposium series – which includes physicians, residents, scientists and students; biotechnology groups; venture capitalists and patients and their families (*see 6. Products*).

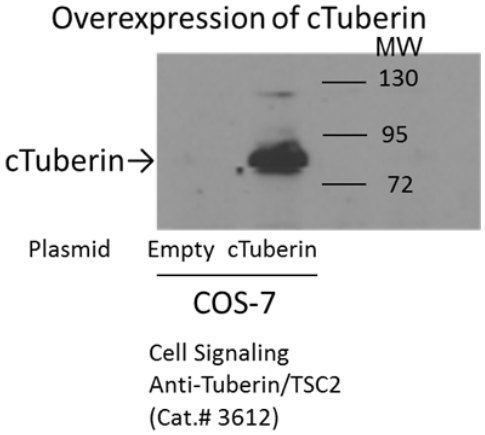


Fig. 4. Overexpression of cTuberin. To test for expression of the newly constructed cTuberin, the DNA plasmid for AAV-CBA-cTuberin tagged with a c-Myc antigenic epitope was transfected into COS-7 cells. Western blotting was performed on cell lysates, using an empty vector as a control. The AAV-cTuberin construct generated an immunoreactive band recognized by anti-Tuberin/TSC2 antibody (# 3612, Cell Signaling Technology, Danvers, MA) of the predicted MW for cTuberin, 85 kD.

What do you plan to do during the next reporting period to accomplish the goals?

We are on target to achieve all our milestones as outlined in the SOW. We anticipate submitting a manuscript for publication on the AAV-hamartin gene therapy within the next 6 months, and will have completed experiments for a second manuscript on AAV-cTuberin gene therapy.

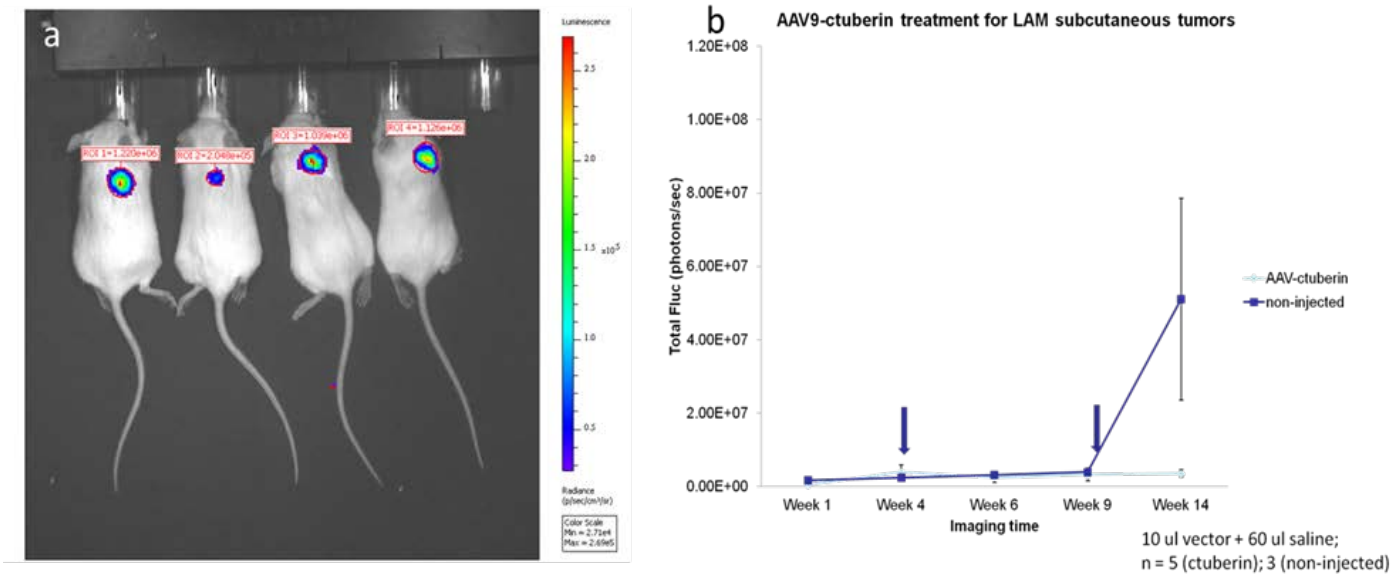


Fig. 5. Monitoring LAM tumor volumes *in vivo* by bioluminescence imaging. a) 3 million TSC2-/- LAM cells stably transduced with lentivirus expressing firefly luciferase were mixed 1:1 with Matrigel and injected subcutaneously into NOD-SCID Il2R gamma (NSG) mice. **b)** tumor volumes were monitored at intervals by *in vivo* bioluminescence imaging. At the time points indicated (arrows; weeks 4 and 9) tumors were injected with either AAV9-cTuberin vector (3 x 10^9 g.c. in 10 μl + 60 μl saline) or not injected.

4. IMPACT

What was the impact on the development of the principal discipline's of the project?

In the course of this work we have generated AAV vectors encoding both hamartin and cTuberin which have been made available to other collaborative investigators for biochemical analyses (Dr. Masao Kaneki and Dr. Vijaya Ramesh, MGH) and will be available to the scientific community upon publication. We have also stably transduced the immortalized TSC2 LAM cells with firefly luciferase to monitor tumor growth by *in vivo* bioluminescence imaging and have provided these to Dr. Elizabeth Henske and David Kwiatkowski, Brigham and Women's Hospital.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

We are in the process of transferring results of our studies to Program Officers at the National Institutes of Health to evaluate next steps in preclinical studies to optimize this therapeutic strategy through the Ignite program.

We have discussed our results with two biotechnology companies under Confidential Disclosure Agreements to probe their interest in working with us to bring this technology to clinical trials.

What was the impact on society beyond science and technology?

Nothing to disclose.

5. Changes/Problems:

Changes in the approach and reasons for change? There are no changes in our approach.

Actual or anticipated problems or delays and actions or plans to resolve them? We do not anticipate any problems or delays.

Changes that had a significant impact on expenditures? No change.

Significant changes in use or care of human subjects, vertebrate animals, biohazards and/or select agents? No changes**.**

6. PRODUCTS

Publications, conference papers and presentations.
Journal/Book publications – none during this period.

Presentation
May 25, 2017 1st Annual Herscot Center TSC Symposium X.O. Breakefield, Invited guest speaker: "Gene Therapy for TSC in Mouse Models"

Website(s) or other Internet site(s) None.

Technologies or techniques – None to report.
Inventions, patent applications and/or licenses – None to report.
Other projects – None to report.

7. PARTICIPANTS and other collaborating organizations

Name: Xandra O. Breakefield, Ph.D. "no change"
Name: Shilpa Prabhakar, M.S. "no change"

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period? No change.

What other organizations are involved as partners? None.

8. SPECIAL REPORTING REQUIREMENTS – Not applicable.

9. APPENDICES

Invention disclosure to Partners Innovation
Title of invention: Gene Therapy for tuberous sclerosis – AAV-CBA-cTuberin
Description of Invention:
Tuberous sclerosis complex (TSC) is a tumor suppressor syndrome inherited in an autosomal dominant manner with an incidence of 1/55,000. Patients inherit a mutation in one allele of the TSC1 gene (encodes hamartin) or the TSC2 gene (encodes tuberin). These proteins together suppress mTORC1 activity. If a mutation in the corresponding normal allele occurs during development or in some somatic cells, it results in enlargement and increased proliferation of cells, forming benign tumors. These tumors can affect a variety of tissues, including brain, heart, kidneys, skin and lungs. In the brain they can cause developmental delay, autism, epilepsy and hydrocephalus. Life threatening conditions in TSC include renal angiomyolipomas, which can cause internal bleeding, and lymphangioleiomyomatosis (LAM), which can compromise breathing. Although rapamycin and related drugs have been effective in reducing the size of lesions for some types of

tumors, they must be administered continuously and have side effect, including compromised brain development and immune suppression. In addition some patients do not respond to these medications, or respond initially and then become resistant.

In previous studies we have shown in a stochastic mouse model of Tsc1 with neurologic involvement (Prabhakar et al., 2013) that we can reduce lesions in the brain and extend lifespan by administration of an adeno-associated virus (AAV) vector encoding hamartin under a strong ubiquitous promoter either by intracerebral ventricular injections (Prabhakar et al., 2015) or intravascular injections (unpublished data, submitted to MGH with a previous invention disclosure for gene therapy for TSC1 using an AAV-CMV-hamartin vector).

The size of tuberin cDNA is 5.4 kb, exceeding the transgene packaging capacity of AAV. The novel aspect of this patent is that we generated a "condensed" version of human tuberin, termed cTuberin which fits into an AAV vector. This was accomplished by deleting the central portion of the cDNA. It was not obvious that using this approach of creating a condensed tuberin that it would be functional, as proper folding and function of modified proteins must be determined empirically. This cTuberin cDNA has been cloned into an AAV vector under a strong ubiquitous CBA promoter. We have transduced mouse embryonic fibroblasts (MEFs) with this AAV-CBA-cTuberin construct and shown by Western blot analysis that it reduces S6 kinase activity, which is a marker of mTORC1 activation, thus establishing biologic activity of this novel protein. Studies are underway to further characterize biologic properties of this protein, e.g. does it bind hamartin and does it have GAP activity toward Rheb, as well as to test its preclinical therapeutic efficacy in mouse models, including xenografts of TSC2 LAM cells and Tsc2 floxed mice injected with the AAVCre vector.

Prabhakar, S., Goto, J., Zuang, X., Sena-Esteves, M., Bronson, R., Brockmann, J., Gianni, D., Wojtkiewicz, G.R., Chen, J.W., Stemmer-Rachamimov, A., Kwiatkowski, D.J., Breakefield, X.O.: Stochastic model of Tsc1 lesions in mouse brain. PLoS One 8:e64224, 2013.

Prabhakar, S., Zhang, X., Goto, J., Han, S., Lai, C., Bronson, R., Sena-Esteves, M., Ramesh, V., Stemmer-Rachamimov, A., Kwiatkowski, D.J., Breakefield, X.O.: Survival benefit and phenotypic improvement by hamartin gene therapy in a tuberous sclerosis mouse brain model. Neurobiology of Disease 82:22-31, 2015.

Distinguishing novel features of invention

We have designed a version of human tuberin which retains the hamartin binding region in the N-terminal and the GAP region in the C-terminal, and lacks critical Akt phosphorylation site Thr1462, such that Akt presumably cannot activate mTORC1. The central region of the protein is replaced by a glycine-serine linker to confer conformational flexibility. Preliminary data indicate that cTuberin is functionally active in suppressing downstream effects of mTORC1 activation.

Envisioned commercial products or processes

AAV vectors have proven safe and beneficial in gene therapy for a number of human diseases, and FDA products are appearing this year. These vectors can be delivered intravascularly to reach many tissues in a single injection, with some serotypes able to cross the blood brain barrier. Typically a single injection confers beneficial outcome over a long term. Our invention will allow use of this vector for treatment of manifestations of tuberous sclerosis in patients with mutations in TSC2. We would initially target renal angiomyolipomas, then LAM and then brain dysfunction. In the case of renal angiomyolipomas, tumor size can be monitored by MRI and the shrinkage in cell size due to replacement of tuberin function can be revealed within months, as it was in the clinical trial for rapamycin carried out by Dr. Elizabeth Thiele at MGH.

cTuberin is anticipated to be safer than rapamycin and related drugs. Rapamycin and related drugs exert their beneficial effects by inhibiting mTORC1 activity in TSC. Rapamycin and related drugs are capable of completely abrogating mTORC1 activity and may reduce mTORC1 activity to levels much lower than the normal levels, which, in turn can cause toxic and/or adverse effects. In contrast, cTuberin is capable of down-regulating mTORC1 activity to the normal level by restoring the regulation of mTORC1 in TSC, but does not completely inhibit mTORC1. It is anticipated that unlike rapamycin and related drugs, cTuberin will not cause toxicity and adverse events related to over-suppression of mTORC1. Further, since tuberin, and by analogy cTuberin, is only active when complexed with hamartin, and hamartin levels are normal in TSC2 patients, there should be little-to-no toxicity due to overexpression of cTuberin through vector delivery.

cTuberin is anticipated to be more efficacious as well as safer than rapamycin and related drugs. TSC2 regulates not only mTORC1 activity but also mTORC1-independent Rheb-dependent pathway. Recently, accumulated evidence indicates that over and above mTORC1 hyperactivation, mTORC1-independent Rheb-dependent pathway plays a pivotal role in the pathogenesis of TSC caused by TSC2 deficiency. Rapamycin and related drugs effectively inhibit mTORC1 activity, but do not restore the regulation of mTORC1-independent Rheb-dependent actions in TSC2 deficiency. In contrast, cTuberin is capable of inhibiting both mTORC1 activity and mTORC1-independent Rheb-dependent pathological actions in TSC2 deficiency.

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Prabhakar S, Goto J, Zhang X, Sena-Esteves M, Bronson R, Brockmann J, Gianni D, Wojtkiewicz GR, Chen JW, Stemmer-Rachamimov A, Kwiatkowski DJ, Breakefield XO (2013) Stochastic model of Tsc1 lesions in mouse brain. PLoS One 8(5): e64224. doi: 10.1371/journal.pone.0064224

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Yu J, Astrinidis A, Howard S, Henske EP (2004) Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and nongenomic signaling pathways. Am J Physiol Lung Cell Mol Physiol 286(4): L694-700